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Immune response to the long-term grafting of cryopreserved small-diameter arterial allografts

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Summary. Introduction. The viability and immunological response induced by cryopreserved arterial allografts remain unclear. This study examines the post-graft behaviour of this type of vessel substitute. Materials and methods. Both iliac arteries were extracted from Lewis rats (donors) and used to establish groups of allogeneic fresh (group I) or cryopreserved (group II) grafts in Fisher-344 rats (recipients). Cryopreserved segments for grafting were prepared by automated controlled freezing at a cooling rate of 1°C/min followed by storage in liquid nitrogen vapour at -145°C for 30 days. Before grafting, the vessels were slowly thawed. Animals were sacrificed at 14, 30, 90 and 180 days post-surgery when graft specimens were obtained for light and electron microscopy and immunohistochemical detection of inflammatory cells (CD45, ED1, CD4, CD8). Results. After surgery, 85.71% of the grafts in group I and 82.14% in group II were patent. Following long-term implant, both the fresh and cryopreserved allografts showed complete loss of the muscle compartment of the media. Inflammatory or CD45-positive cells (mainly macrophages and CD8 T-lymphocytes) were detected at earlier time points in suture zones and adventitia. In the fresh allografts, the number of immunolabelled cells steadily increased until they were seen to occupy the entire adventitia at 90 days, with high numbers persisting at 6 months. In the cryopreserved allografts, this adventitial inflammatory infiltrate was significantly reduced. Conclusions. The cryopreservation/slow thawing protocol used diminished the immune response induced by fresh arterial allografts improving their behaviour after grafting.

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Introduction

Arterial disease is among the topics that has generated most intense research because of the numerous factors that contribute to graft failure. In addition, the rise in the number of bypass surgery procedures conducted, mainly because of the increased life span of the population, has further prompted research in this area.

The surgical substitution of small calibre vessels (under 6 mm) has always been a problem for the vascular surgeon. For bypass procedures, the use of a prosthetic graft is a good alternative to autologous tissue, although these substitutes induce a high incidence of thrombosis and/or infection (Teebken et al., 2004; Batt et al., 2008) and often need to be replaced. An allogeneic arterial graft could be a viable option for this purpose, although their use has been limited by the immunological response produced in the host and the difficulty in preserving these grafts from the time of harvesting to grafting. Recent advances in cryogenics have provided clinicians with a new source of vessel substitutes in the form of a cryopreserved vessel.

The storage of tissue in banks requires the use of cryogenic techniques, and the cryopreservation and subsequent thawing of different tissues has been the subject of numerous investigations (Gournier et al., 1995; Pascual et al., 2001; Müller-Schweinitzer et al., 2007). The best method of freezing human tissue is an automated, gradual process and this procedure has been well standardised. However, the most adequate storage temperature or thawing protocol have not yet been defined.

The difficulties faced when following the post-implant course of a human vascular graft has made it

difficult to determine the real cause or the events that eventually trigger the failure of the grafted vessel. The issue has been therefore addressed in animal models. In prior studies performed in autologous or syngeneic vessel transplant models, we were able to follow the tissue repair, or scarring process, in the absence of an immune response (Bellón et al., 1996; Pascual et al., 2004).

When an allogeneic model is used, we may expect an immune response produced in the recipient animal. According to several authors, the immunogenicity of a vessel is mainly attributable to its cell compartment, since the extracellular matrix on its own is incapable of inducing an acute immune reaction or a chronic rejection process (Allaire et al., 1994; Plissonnier et al., 1995; Häyry, 1998; Fellström et al., 1999). Studies by Pilssonnier et al. (1995) and Matia et al. (2007) have suggested that the cell invasion of the adventitia could be due to the presence of antigens in the medial layer, and that this response continues until the cell component disappears. According to the results obtained by these authors, the disappearance of the cell component and necrosis of the media seems to be dependent on the presence of immunoglobulins. Both the ischaemia-reperfusion process to which vascular grafts are subjected to, and incompatibility between donor and recipient in terms of the major histocompatibility complex (MHC) on the surface of endothelial and muscle cells, promote the adhesion and infiltration of T-lymphocytes, monocytes and neutrophils in the arterial wall (Akyürek et al., 1998), these last two cell types being the first to cross the wall (Rogers et al., 1996; Roque et al., 2000; Welt et al., 2000). In addition, platelets deposited on the lumen wall following endothelial denudation contribute to the adhesion of neutrophils. These cells play an important role in the development of intimal hyperplasia and the progression of restenosis since they induce the release of growth factors and cytokines, stimulating the repair process and contributing to the proliferation and migration of smooth muscle cells (Simon et al., 2000; Jurado et al., 2002).

Despite the current use of cryopreserved allogeneic vessels for particular situations, such as arterial reconstructions in patients with no available autologous vessels, or to replace an infected area with acceptable outcomes (Brown et al., 2009; Vardanian et al., 2009), their long term behaviour is as yet unknown, and there is also controversy over the viability and immune response induced by this type of vessel substitute. Some authors argue that cryopreservation diminishes the immunogenicity of vascular allografts (Deaton et al., 1992; Giglia et al., 2002), while others have observed that the immune response in cryopreserved tissue is similar to that produced in fresh tissue (Moriyama et al., 2000; Gabriel and Fandrich, 2002; Saito et al., 2006).

This study was designed to gain further insight into the long term behaviour of this type of vessel substitute through the use of a model in the rat employing allogeneic iliac artery grafts previously cryopreserved and subjected to a controlled slow warming process, as described by our group (Buján et al., 2001).

Materials and methods

Female Lewis rats (n=28) and female Fisher 344 rats (n=56) weighing 220-250 g were used as donors and recipients respectively. All animals were handled according to European Union guidelines for the care and use of laboratory animals (European Directive 609/86/CEE and European Convention of Council of Europe ETS123).

Graft harvesting

The rats were anaesthetised by the intraperitoneal administration of ketamine chlorhydrate (Ketolar, Parke-Davis, Spain) at 0.25 ml/100 g body weight and diazepan (Valium, Roche, Spain) given as 0.004 ml/100 g body weight.

Surgery was performed with the use of a Wild M-650 surgical microscope. Donor rats were subjected to a midline laparotomy to expose both common iliac arteries. The arteries were dissected along their entire course, resected from the iliac vein, washed in physiological saline to remove blood and placed in MEM (minimal essential medium) (Gibco, Invitrogen, USA) until the moment of grafting as fresh or cryopreserved allogeneic vessel substitutes.

Cryopreservation and thawing

For cryopreservation, iliac artery segments were placed in cryotubes containing MEM supplemented with 20% foetal bovine serum (FBS) (Gibco, Invitrogen, USA) and 10% dimethylsulphoxide (DMSO) (Sigma, USA). The DMSO was added gradually in 4 x 5 minute-steps (to give 2.5, 5, 7.5 and 10% dilutions of DMSO). The vessels were then subjected to automated, controlled freezing in a biological freezer (CM25 P.115, Carburos Metálicos S.A.) to -120°C at a temperature reduction rate of 1°C/minute.

The frozen arterial segments were stored in liquid nitrogen vapour at -145°C for 30 days. After this storage period, specimens were transferred from the storage tank to the freezer where they were thawed according to a slow thawing programme to room temperature at a warming rate of 1°C/minute.

Once thawed, the cryoprotectant was removed gradually in tapered dilutions and the cryografts implanted in the recipient rats.

Grafting procedure

The right common iliac artery in the recipient was dissected and clamped with a double Gilbert microclamp. A portion 5 mm in length was resected and directly substituted in-line with a fresh or cryopreserved arterial segment of the same length by end-to-end

anastomosis through the placement of interrupted sutures of Ethilon 10/0 monofilament thread (Ethicon®).

The grafting and blood recirculation procedure determined a cold ischaemia time of about 35-40 minutes. No anticoagulants or immunosuppressive drugs were administered to the animals.

Experimental design

Two study groups of allogeneic fresh (Group I, n=28) and allogeneic cryopreserved (Group II, n=28) grafts were established.

At 14 (n=7), 30 (n=7), 90 (n=7) or 180 (n=7) days post surgery, the rats were anaesthetised (as described above). Implanted grafts were carefully exposed and the vascular bed perfused first with Ringer's lactate to wash out all the blood, and then with a 2:1 mixture of glutaraldehyde (3%)-paraformaldehyde (2%) in Millonig buffer (pH 7.3), to avoid tissue retraction and achieve good fixing. After the perfusion process, the grafts were harvested leaving margins of the native artery at both ends.

Morphological study

The morphology and ultrastructure of the explanted allografts were examined by light and electron microscopy (transmission and scanning). For light microscopy, the grafts were fixed by immersion in Bouin's fluid and embedded in paraffin. Longitudinal and cross-sections 5 μm-thick were prepared and stained using haematoxylin-eosin, orcein and Masson's trichrome.

For transmission electron microscopy (TEM), small vessel fragments were fixed for 2 hours in 3% glutaraldehyde, stored in Millonig buffer (pH 7.3) and postfixed in 2% osmium tetroxide. Once dehydrated in a graded series of acetone, the specimens were embedded in Araldite to obtain thin cuts. These sections were counterstained with lead citrate and examined using a Zeiss 109 transmission electron microscope.

For scanning electron microscopy (SEM), the harvested grafts were opened longitudinally and immersed in 3% glutaraldehyde. Next, they were transferred to Millonig buffer (pH 7.3) for 1 hour and dehydrated in a graded acetone series reaching critical point in a Polaron E 3000 instrument with CO_2. Once metallized with palladium-gold, the specimens were examined using a Zeiss 950 DSM scanning electron microscope.

Morphometry

Morphometric analysis of the neointimal, medial and adventitial arterial wall layers was conducted on digitalized images of 20 histological sections per graft specimen stained with haematoxylin-eosin, orcein and Masson's trichrome. Images were captured using a digital camera fitted to the microscope, Axiocam HR, (Zeiss) and analysed using image analysis software Axiovision AC 4.1 (Zeiss).

The thicknesses of the different arterial wall layers were measured as follows: intima, from the vessel lumen to the internal elastic lamina (IEL); media, from the IEL to the external elastic lamina (EEL); and adventitia, from the EEL to the end of the tissue.

Immunohistochemical analysis

For the identification of cells, specimens were fixed by immersion in Bouin's fluid, embedded in paraffin and cut into 5 μm-thick sections.

We used monoclonal antibodies directed specifically against the rat leukocyte common antigen CD45 (1:50) (OX-1, BD Biosciences, NJ, USA), monocytes/macrophages-ED1 (1:100) (MCA-341, Serotec, Oxford, UK), CD4 (1:50) (W3/25, CTS 515G, Labgen, LabClinics, Barcelona, Spain), and CD8 lymphocytes (1:50) (MRC OX-8, CTS 418G, Labgen, LabClinics, Barcelona, Spain). As a negative control of the technique used, tissue specimens were incubated with a negative isotype (IgM) of the corresponding primary antibody. Positive controls were lymph node cross-sections. The antigen-antibody reaction was detected by the peroxidase-antiperoxidase-diaminobencidine or phosphatase alkaline-fast red method. Cell nuclei were stained with haematoxylin. All processed specimens were observed under a Zeiss Axiophot light microscope. Percentages of cells positive for the antibodies tested were calculated on 20 histological sections per specimen by counting the numbers of labelled and non-labelled cells in several microscopy fields (400x) selected at random.

Statistical analysis

All statistical tests were performed using the Statgraphics plus program, version 5.1 for Windows. Data were compared using the Mann-Whitney U-test and expressed as means± standard deviation. The level of significance was set at $p<0.05$.

Results

During the postoperative course, no signs of infection or behavioural changes were observed in the animals. Neither were evident signs of acute graft rejection detected.

Of the 56 vascular substitutes, fresh and cryopreserved, 47 (83.92%) were still viable at the time the grafts were harvested. The patency rate was 85.71% for the fresh and 82.14% for the cryopreserved allografts.

In the fresh (group I) allografts, the wound repair process was characterised by the appearance of a proliferative and immature neointimal layer with a dense inflammatory infiltrate, degeneration of the native medial layer and development of a proliferative

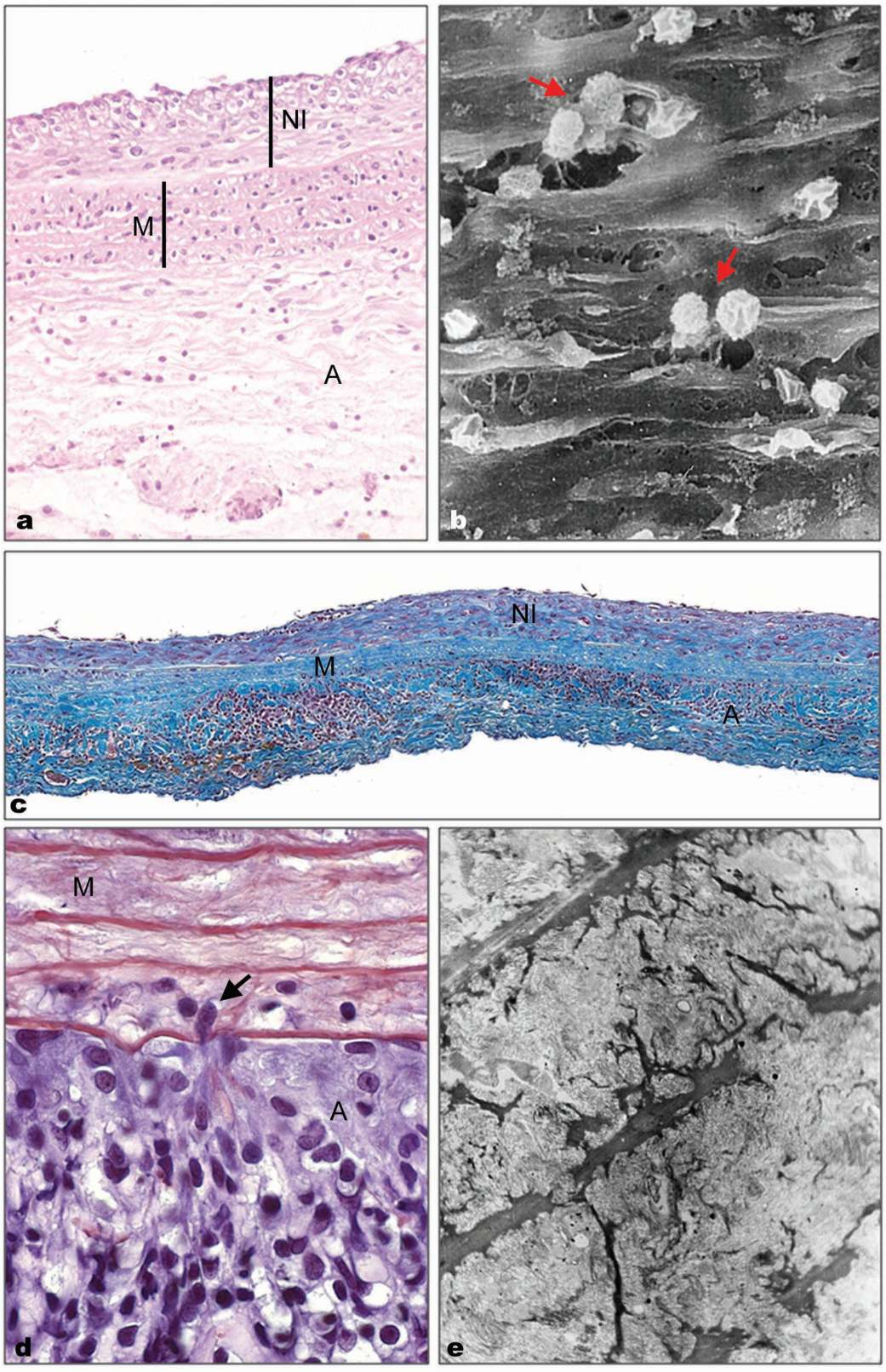

Fig. 1. Fresh allogeneic micrografts. **a.** Image shows the distal zone of an allograft 14 days after grafting in which the variable thickness of the neointima may be noted. **b.** Scanning electron micrograph of the luminal surface of an allograft at 30 days post-graft in which adhered white blood cells (arrows) and loss of intercell junction continuity may be seen. **c.** Medial zone of an allogeneic vascular graft 90 days after implant. **d.** Intense inflammatory infiltrate in the adventitia. Cells penetrate towards the deeper layers of the media (arrows) via fenestrations in the elastic laminae. **e.** In the media, elastic laminae show degenerative signs characterized by their irregularity. NI: neointima, M: medial layer, A: adventitia. a, x 500; b, d, x 1,000; c, x 200; e, x 3,000

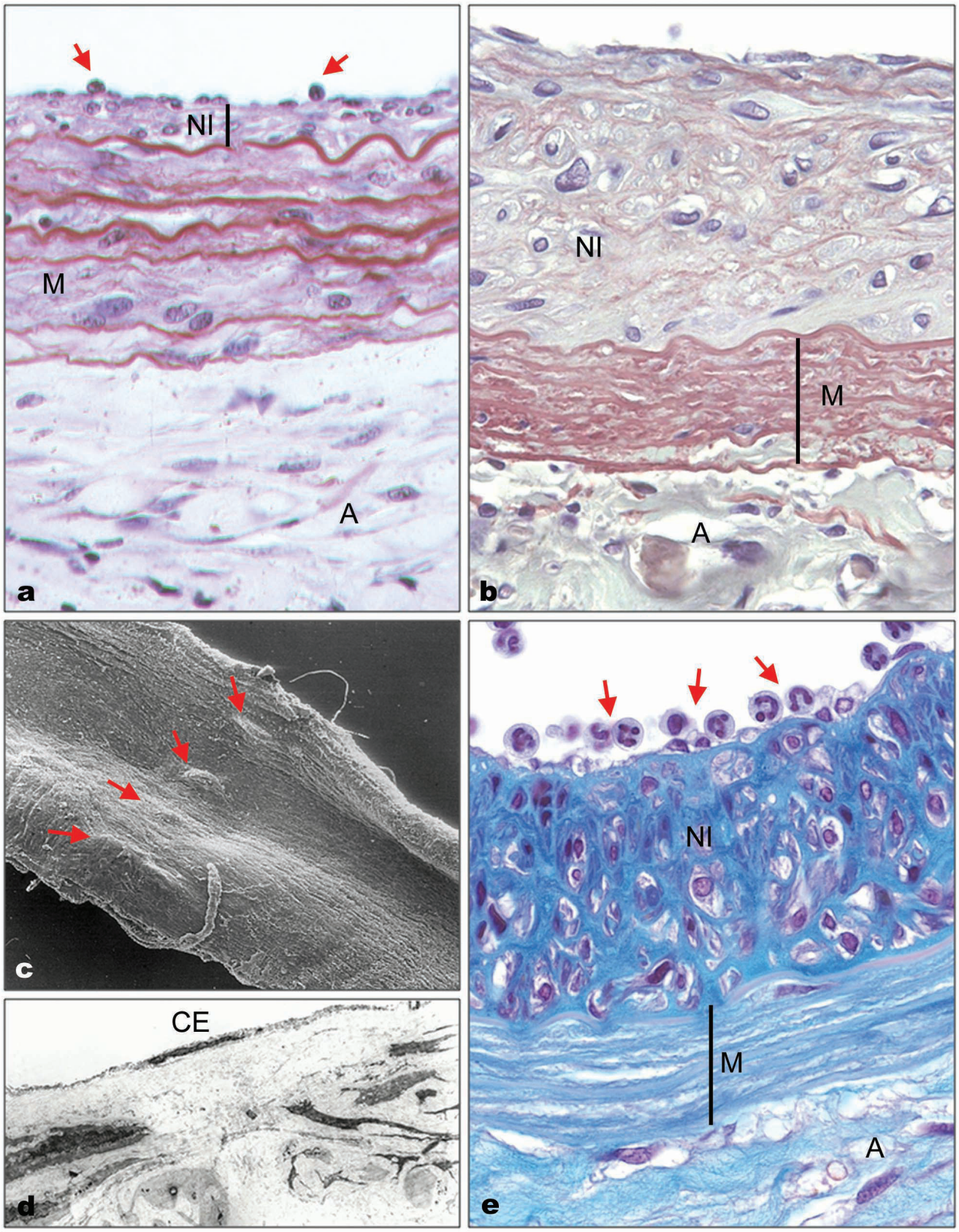

Fig. 2. Cryopreserved allogeneic micrografts. **a.** Presence of white blood cells adhered to the surface of the neointima (arrows) and notable loss of the medial layer's cell component at 14 days post-graft. **b.** Cross section of a 90-day cryopreserved allograft. **c.** Scanning electron micrograph of the proximal anastomosis at 90 days (arrows, suture). **d.** Endothelial cells (EC) and subendothelial matrix in the luminal zone of the neointima. **e.** Presence of numerous polymorphonuclear cells (arrows) on the luminal surface at 6 months post implant. NI: neointima, M: medial layer, A: adventitia. a, x 630; b, x 200; c, x 50; d, x 3,150; e, x 1,000

neoadventitia (with intense neoangiogenesis and fibrosis). Re-endothelialization of the grafted vessel segment was incomplete at each of the follow up times, with denuded zones showing adhered platelets and white blood cells observed in every graft. The thickness of the neointima increased gradually until post-implant day 90, at which time it appeared to stabilize. In the long-term (180 days), this layer seemed to be made up of white blood cells and smooth muscle cells embedded in an active extracellular matrix. The grafts triggered a substantial inflammatory reaction, which peaked at 90 days (Fig. 1).

In group II, the vessel remodelling process observed was similar to that noted in the fresh allografts, although cell loss in the medial layer was evident at 14 days. This type of vessel substitute induced a significantly reduced immune response at all the follow up times (Fig. 2).

Morphometry

The appearance of a neointimal layer caused significant wall thickening in the fresh allografts. At 90 days, the arterial wall had attained its maximum thickness despite gradual thinning of the medial layer. This thickening was due to an increased thickness of the neointimal and adventitial layers caused by fibrosis and the presence of inflammatory cells. This process seemed to stabilize between 90 and 180 days.

Following the implant of the cryopreserved grafts, we observed a recovered wall thickness compared to the control cryopreserved iliac artery. Progression of the neointimal layer was similar to that detected in the fresh allografts, although it was slightly thinner with significant differences appearing at 14, 30 and 90 days. An important difference was the rapid involution of the media, with evident thinning produced at 14 days, resulting in significant differences between the two groups up until 90 days. In contrast with the fresh allografts, the thickness of the tunica adventitia did not suffer major changes. At 6 months, the thickness of the neointima and media were similar in the two graft types (Fig. 3).

Immunohistochemistry

Labelling of the common leukocyte antigen CD45 indicates the general presence of white blood cells. In the group I allografts, this antigen was moderately expressed at the initial time points and positive cells were mainly observed in zones close to the sutures and isolated through the rest of the adventitia. Thereafter, the number of labelled cells steadily rose until the entire adventitia was occupied at 90 days, and these peak levels persisted up to the 6-month time point. In the remaining arterial layers, cells positive for CD45 sporadically appeared. In group II, most CD45-positive cells were detected at the level of the neointima and a significant reduction compared to group I was noted in the adventitia (Fig. 4).

When specific white blood cell markers were used, positive cells were mainly identified as macrophages and lymphocytes, their percentages being significantly lower in the cryopreserved grafts.

In group I, the presence of macrophages was particularly intense at 2 weeks in the suture zones and on the graft luminal surface. At 30 days, their presence increased and these were mainly found in the tunica adventitia and neointima, observing a few isolated macrophages in the media. ED1 cells peaked at 90 days and high numbers persisted up until 180 days (Fig. 5a,c,e). Cells positive for the CD8 T antigen mainly appeared at the level of the adventitia, with only a few isolated cells observed in the neointima. The presence of CD4 T-lymphocytes was inversely proportional to that of the CD8 T-lymphocytes. We were only able to detect a few sporadic labelled cells in the adventitia or neointimal surface (Fig. 6a,c,e).

In the group II grafts, macrophages (Fig. 5b,d) and lymphocytes (Fig. 6b,d) were mainly observed in the neointima and in zones close to anastomoses. Both the percentage of ED1 cells and lymphocytes was significantly lower than observed in the fresh grafts due to the scarce presence of macrophages in the adventitia (Figs. 5e, 6e).

Discussion

The viability of arterial allografts has been variably described in the literature. For example, Hancock et al. (1995) reported a drop in patency as their study time increased to reach a rate of 50% in the long term.

Previous results published by our group (Pascual et

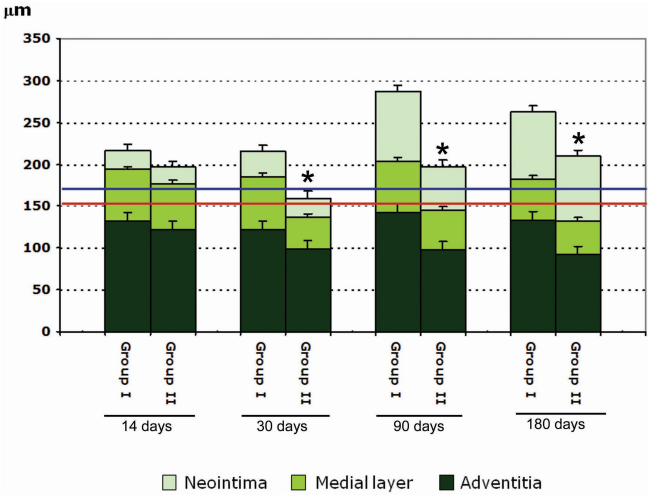

Fig. 3. Arterial wall thicknesses recorded in each of the study groups. Blue line indicates the mean wall thickness of a normal iliac artery and the red line, the mean thickness of a control arterial segment after cryopreservation. Differences were detected in overall wall thicknesses recorded in the fresh versus cryopreserved allografts on post-graft days 30, 90 and 180 ($p<0.05$).

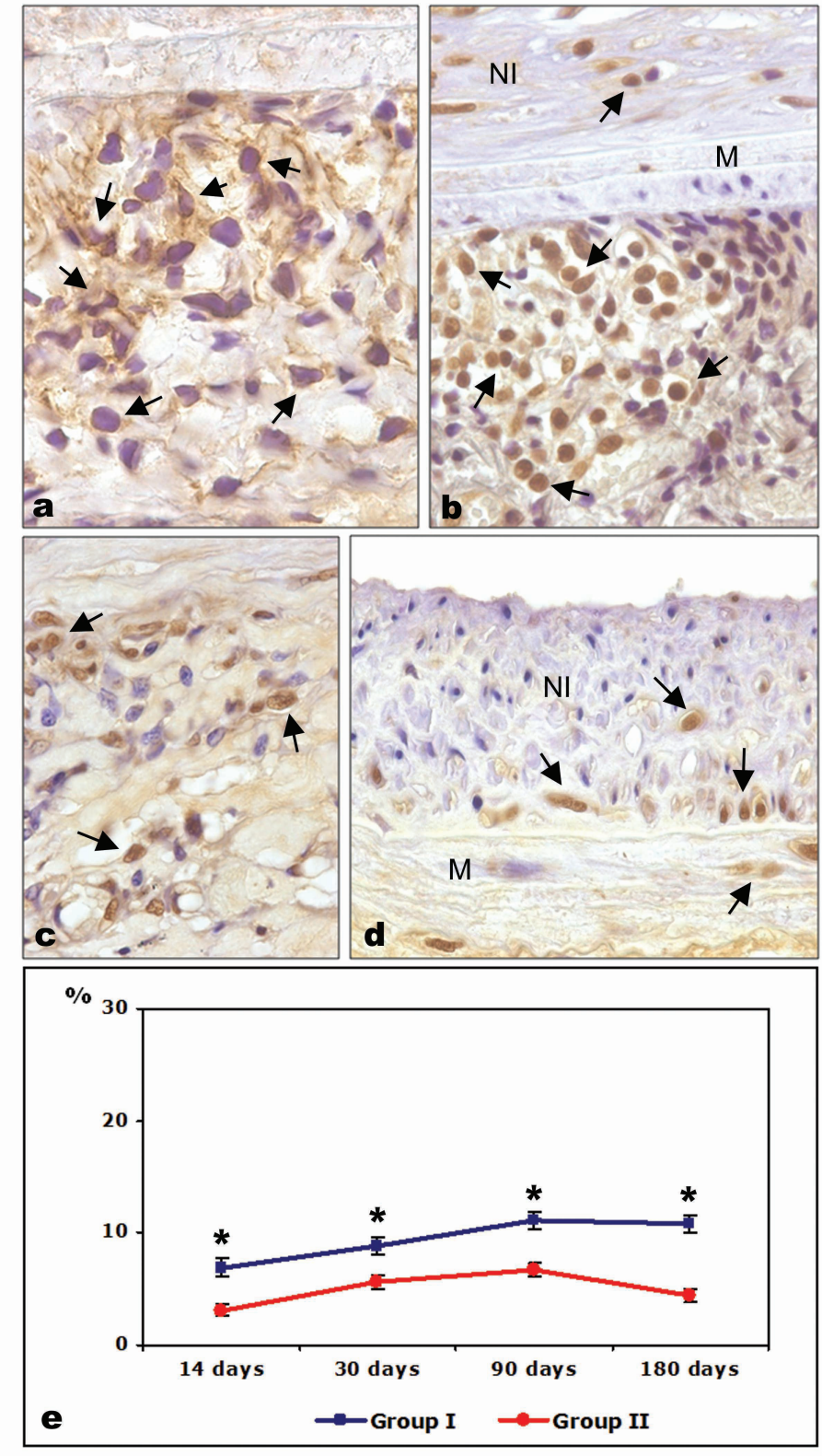

Fig. 4. Expression of CD45 (arrows). **a.** Intense labelling of inflammatory cells of the adventitia in fresh allografts at 90 days. **b.** CD45-positive cells are still numerous in the fresh allografts at 6 months. **c.** Zone close to the anastomosis in a cryopreserved allograft at 90 days. **d.** Cryopreserved allograft at 180 days, in which white blood cells infiltrated in the neointima and medial layer appeared labelled. **e.** Percentage of CD45-positive cells in the vessel wall of the implanted allografts. Significant differences were observed between the two study groups at all the follow up times ($p<0.05$). NI: neointima, M: medial layer. a, c, x 1,000; b, d, x 630

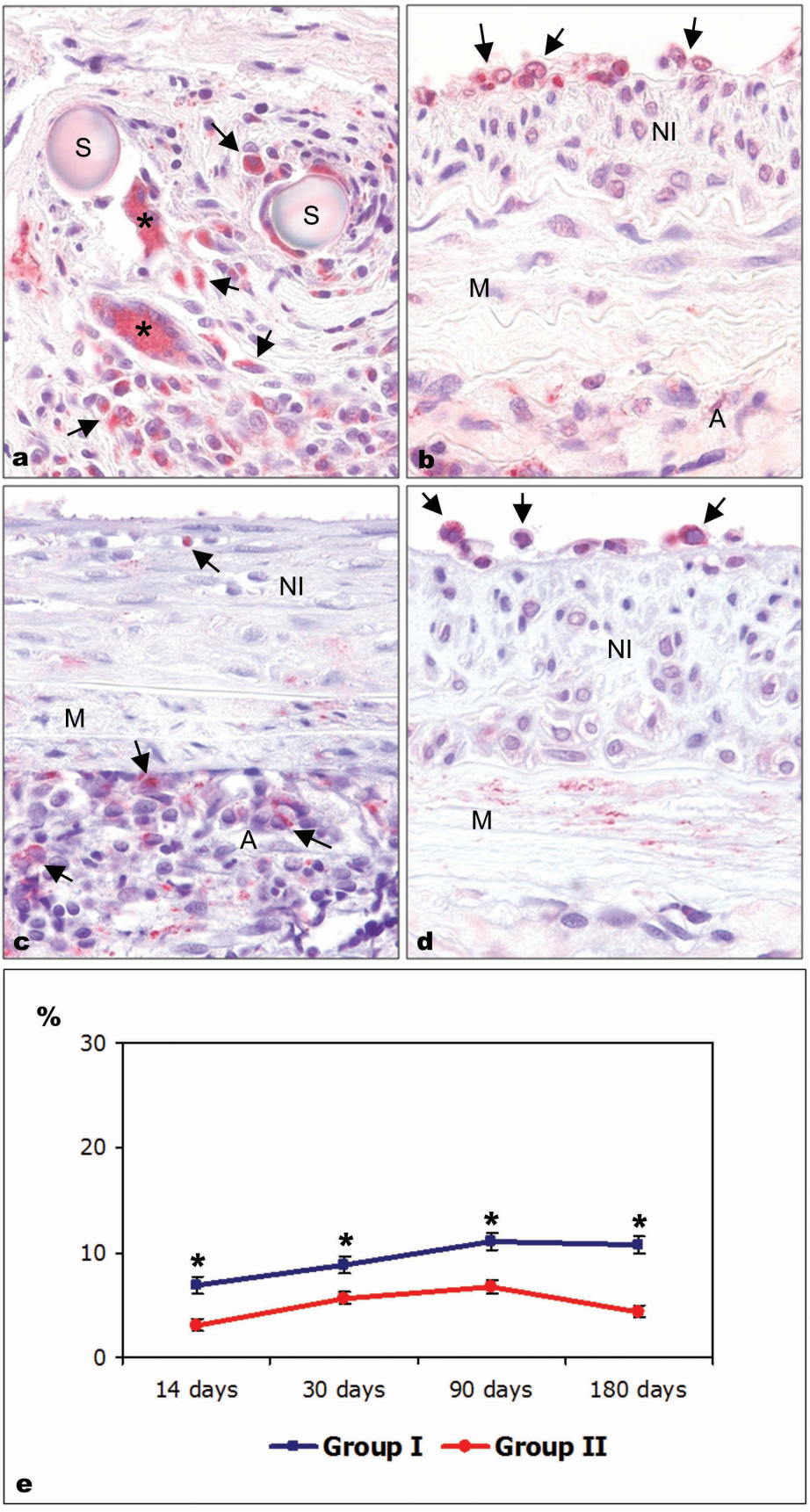

Fig. 5. Expression of ED1 (arrows). **a.** Macrophages and giant foreign body cells (*) around the suture stitches in a fresh allograft at 30 days. **b.** Cryopreserved allograft at one month post-implant. **c.** Labelling remained intense in the neointima and adventitia at 180 days post-graft in the fresh allografts. **d.** At 6 months, positive cells were only observed on the luminal surface of the cryograft. **e.** Proportions of ED1-positive cells (monocytes/macrophages) detected in the grafted vessel segments ($p<0.05$). S: suture, NI: neointima, M: medial layer, A: adventitia. a, c, x 630; b, d, x 1,000

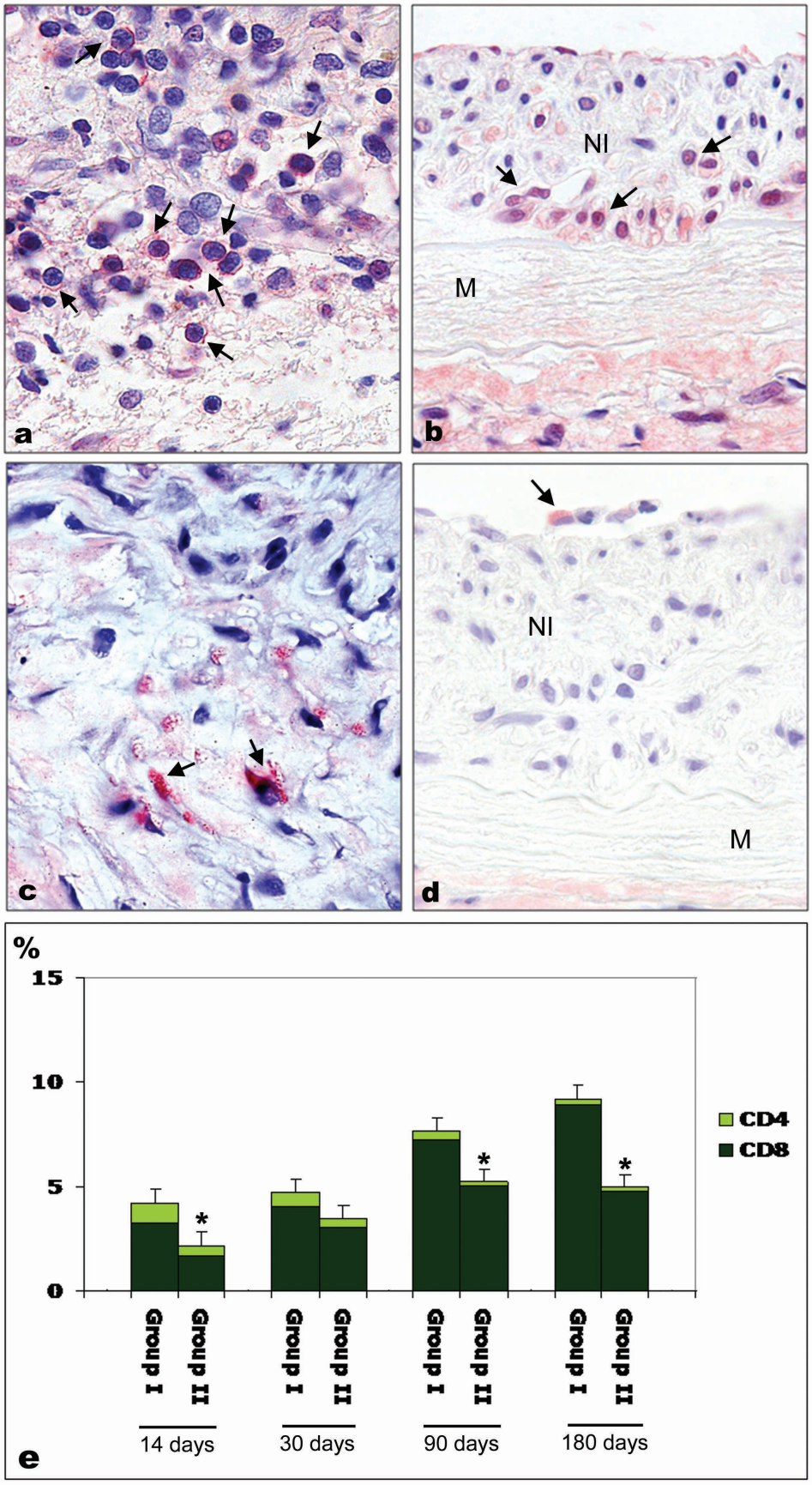

Fig. 6. a. Expression of CD8 T-cells (arrows) in the adventitia of the fresh allografts implanted for 180 days. **b.** Lymphocytes infiltrating the lower third of the neointima in a cryopreserved graft at 180 days. **c.** Discrete presence of CD4 T-lymphocytes (arrows) in the fresh allografts at 14 days. **d.** Distribution of CD4 T-lymphocytes in a cryopreserved allograft at 180 days. **e.** Percentages of CD4 and CD8 T-lymphocytes recorded in each of the study groups (*p<0.05). NI: neointima, M: medial layer. a, d, x 630; b, x 400; c, x 1,000

al., 2004) have indicated that following the cryopreservation and thawing of iliac artery substitutes minimal histological changes occur. These include well preserved cellularity of the medial layer despite some randomly distributed areas lacking endothelium on the luminal surface of the arterial wall, making them good candidates for vessel substitutes.

When comparing the two types of allograft (fresh and cryopreserved), some authors (Takeishi et al., 1994; Mesa et al., 1997) have reported better results for cryopreserved allografts at 3 months post implant. However, Nataf et al. (1995) obtained similar patency rates for the two graft types using carotid artery grafts in sheep. Komorowska-Timek et al. (2002) also described similar results for fresh and cryopreserved femoral artery allografts 4 months after transplant from Fischer to Wistar rats. Notwithstanding, longer implant times (8 months) had a dramatic impact on patency, and while all the fresh allografts remained patent, the cryografts were all thrombosed. We recorded similarly high patency rates here at 6 months for both our fresh (85.71%) and cryopreserved allografts (82.14%).

The tunica media is the layer that contains the larger cell population, which should be a substantial source of antigens (Salomon et al., 1993). However, the immune response is not as intense as might be expected. In contrast, the adventitia shows few cell components, which could suggest its low immunogenic capacity, yet it is the zone showing the greatest inflammatory reaction. The adventitial response in the fresh allografts was very active, especially at 90 days. The presence of lymphoid cells, the increased neoangiogenesis and fibrosis differ from observations in the neoadventitia formed as a consequence of the repair process induced by graft placement.

In our model, the inflammatory response was significantly greater in the fresh than the cryopreserved grafts. This response was measured in terms of the numbers of CD45, CD4, CD8 and ED1 cells observed. The major presence of CD8 over CD4 T-lymphocytes indicates negative regulation of the repair process (Regan and Barbul, 1991; Légare et al., 2000). Biedermann and Pober (1999) showed that antigens of an allogeneic nature activated a single population of cytotoxic CD8 T-lymphocytes that were less efficient at destroying target cells.

The lack of CD4 T-cells observed here could be attributed to our longer term study points since we did not examine the acute rejection stage. Our observations are consistent with the triggering of a long-term immune response or chronic rejection process due to the persistence of donor antigens.

The loss of the cell component of the medial layer or appearance of intimal hyperplasia have been attributed, in early studies addressing the use of allografts, to the immune response (Takheishi et al., 1994; Akyürek et al., 1998). In studies performed by Plissonier et al. in 1993 and 1995, and by Matia et al. in 2007 on the rejection of vascular grafts in rats, a correlation was observed between the disappearance of medial layer cells and the inflammatory reaction produced in the adventitia, such that this process continued while there were still cells in the media. These authors also demonstrated the presence of immunoglobulins in the medial layer. Thus, these could be involved in the late inflammatory response that takes place in allografts. Nataf et al. (1996) and Lehr et al. (2011) also noted that removal of the cell compartment avoided the inflammatory reaction of the adventitia in allogeneic grafts.

According to the theory of Plissonnier et al. (1993, 1995) Matia et al. (2007) and Nataf et al. (1996), it could be that the loss of the cell compartment of the medial layer diminishes the number of exposed antigens, and thus the inflammatory reaction. Perhaps for this reason, cryopreserved grafts, which show earlier decellularization of the medial layer, generate a more discrete inflammatory response than allografts not subjected to cryopreservation, in which the disappearance of the cell component takes place between 30 and 90 days.

However, this event has also been observed in other vessel substitution models. The response of the arterial wall to insult gives rise to a wound repair process that, regardless of the type of substitute (autogeneic or allogeneic, fresh or cryopreserved), will lead to similar outcomes in the long term. Thus, sooner or later, all arterial fragments will be transformed into elastic conduits with a decellularized medial layer surrounded by a fibrous capsule, in which the neointima may take on some of the functions of the media (Bellón et al., 1996; Pascual et al., 2005).

Reports in the literature reveal the high variability in the time sequence of immune response events. This could be explained by the high variability also in the experimental animals used. Hence, several authors report a great inflammatory reaction at early time points that persists for 30 or 60 days (Mennander et al., 1991; Hirsch et al., 1998; Gabriel et al., 2006), while others describe a later response (Bojakoswki et al., 2000; Religa et al., 2003) and a behaviour similar to that observed here in which the immune response continues beyond this time interval.

Our findings indicate any arterial segment used in revascularization surgery will progress towards the formation of a fibrous neovessel wall, in such a way that if this substitute contains elastic laminae and is poorly antigenic, it will persist in the recipient as an elastic conduit offering certain compliance at the graft site. Compared to a fresh allograft, a cryopreserved allograft will show some immunogenicity yet will elicit a more attenuated rejection process.

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